APPENDIX B

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Wolfgang Woloszczuk *et al.*

Serial No.: 10/596,968

Filed: June 30, 2006

For: IDENTIFICATION OF FELINE OR CANINE proBNP

Confirmation No.: 3302

Group Art Unit: 1641

Examiner: FOSTER, Christine E.

Atty. Dkt. No.: SONN:093US

CERTIFICATE OF ELECTRONIC TRANSMISSION

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

May 18, 2010
Date

Travis M. Wohlers

DECLARATION OF GIOSI FARACE UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

I, Giosi Farace, the undersigned, declare that:

1. I am a Research Scientist II at Idexx Laboratories, Inc. I have extensive research experience in protein detection and antibody-based diagnostic assays. A copy of my *curriculum vitae*, listing my publications in this regard, is attached as Exhibit A.

2. I am providing this declaration to describe certain studies I performed at Idexx Laboratories regarding the detection of NTproBNP. These studies were performed to assess the species specificity of antibodies against NTproBNP.

3. In one study I investigated whether human NTproBNP can be measured using an assay kit for canine NTproBNP. Human glycosylated NTproBNP (cat. no. 8NT1, Lot 05/11-8TN1, Hytest Ltd., Finland) and human non-glycosylated NTproBNP (cat. no. 8GOB2, Lot 07/06-8GOB2, Hytest Ltd., Finland) were diluted serially in BNP-free human plasma (cat. no. 8FBP, Lot 06/01-8BFP, Hytest Ltd., Finland) and assayed using the VetSign Canine Cardioscreen NTproBNP kit (product VC4010, Guildhay Limited, Guildford, England). Serial dilutions ranged from 100 ng/ml to 0.78 ng/ml as indicated. The human samples were assayed in triplicate, and the data were fitted to a linear curve. *See* Exhibit B. The assay protocol used was generally per the kit insert, *see* Exhibit D, except for any changes noted in Exhibit B. As described in Exhibit D, the assay utilizes two sheep polyclonal antibodies with the capture antibody being directed to amino acids 25-41 of the canine NTproBNP molecule and a detection antibody directed against amino acids 1-22 of the canine NTproBNP molecule. The studies reported in Exhibit B demonstrated that canine antibodies failed to recognize human NTproBNP whether or not it is glycosylated. These results indicate that human and canine NTproBNPs are structurally different from one another.

4. In another study I investigated whether canine NTproBNP antibodies can recognize feline NTproBNP, and vice versa. Calibrators from a kit specific for canine NTproBNP (product KE970, IDEXX Laboratories, Inc., USA) were assayed using a kit specific for feline NTproBNP (product VC5010-9G, Biomedica Gruppe, Austria). Second, calibrators from the feline-specific kit were assayed using the canine-specific kit. The data were fitted using a quadratic curve. *See* Exhibit C. The experimental protocols used for this experiment followed the IDEXX SOP for running the assay (*see* Exhibits E and

F). Any deviations from the protocols are described in Exhibit C. The canine assay utilizes two sheep polyclonal antibodies with the capture antibody being directed to amino acids 25-41 of the canine NTproBNP molecule and a detection antibody directed against amino acids 1-22 of the canine NTproBNP molecule. The feline assay utilizes two sheep polyclonal assays with the capture antibody being directed against amino acids 1-20 of the feline NTproBNP molecule and the detection antibody directed against amino acids 60-80.

5. The studies reported in Exhibit C demonstrated that feline antibodies failed to recognize canine NTproBNP, and canine antibodies failed to recognize feline NTproBNP. These results indicate that canine and feline NTproBNPs are structurally different from one another.

6. Together, these studies demonstrate that NTproBNPs differ structurally across species, and that the antibodies involved in these studies are species-specific.

7. I declare that all statements made herein of my own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code.

April 26th 2010
Date

Giosi Farace
Giosi Farace

EXHIBIT A

Dr. Giosi Farace

Contact Details

Address: 98 Knubble Road
Georgetown, ME
04548

Telephone: 207-556-8528 (work)

Email: giosi-farace@idexx.com

Employment History

May 2004 – Present

Research Scientist
IDEXX Laboratories, Inc

I have been involved in novel instrument and assay development projects aiming to validate or transfer novel biomarkers into the veterinary space. These projects have mainly focused on the areas of endocrinology and cardiology.

Apr. 1999 – Oct. 2002

Postdoctoral Research Associate
University of Manchester &
Queen Mary, University of London

I worked on collaborative multi-country project funded by the European Union aiming to develop a novel electrochemical biosensor platform for the detection of food borne pathogens. My role in the project was technically to develop a system to detect DNA hybridization events using impedance spectroscopy. Administratively I was involved in coordinating the efforts of the other partners in the project.

Aug. 1995 – Dec. 1995

Internship
Thrombosis Research, Ciba Pharmaceuticals

I worked on a project using surface plasmon resonance to monitor part of the coagulation cascade.

Apr. 1993 – Sept. 1993

Internship
Albert Soiland Cancer Research Laboratory, USC

Helped initiate and coordinate an animal study investigating chorodial melanoma.

Apr. 1992 – Sept. 1992

Internship
Institute of Cancer Research

I worked on an ongoing project evaluating novel platinum anti-cancer compounds.

Apr. 1991 – Sept. 1991

Internship
Ministry of Agriculture Fisheries and Food

I worked alongside the staff of this government department collecting and analyzing crop samples.

Dr. Giosi Farace

Education History

1996-2000

University of Nottingham, Nottingham, UK
Ph.D. Biophysical Applications of Near-Field Scanning Optical Microscopy and the Development of Protein Micro-Patterns

My Ph.D. studies covered two broad themes. The first being the evaluation of a novel biophysical tool – near field scanning optical microscopy and the second the creation of patterned arrays of proteins on carbon and polymeric surfaces for ultimate use in tissue culture or biosensor applications.

1995-1996

University of Leicester, Leicester, UK
Msc. Biomolecular Technology

This was a masters program concerning the use of analytical biophysical tools and their applications to the life sciences.

1990-1994

Brunel University. London, UK
BSc. (Hons) Applied Biochemistry

Patents Awarded

2009

US7566573 Dual standard curve immunoassay

Grants Awarded

2003

In situ fabricated membranes for selectivity and biocompatibility in mcroanalytical systems (value £363,400)

Publications

2005

Gill A., Lillie G., Farace G., Vadgama P. Biocompatible interfaces for biosensors. International Journal of Environmental Analytical Chemistry 85, 699-725.

Ahmed S., Dack, C., Farace, G., Rigby, G,. Vadgama P. Tissue implanted glucose needle electrodes: early sensor stabilization and tissue-blood correlation during the run in period. Analytica Chimica Acta 537, 153-161

2004

Farace G., Vadgama P. Bioanalytical application of impedance analysis: transducing in polymer-based biosensors Chapter in Ultrathin Electrochemical Chemo- and Biosensors, Technology and Performance, Part III: Non invasive electrical monitoring in living cells (Volume Editor Mirsky VM,) 181-198.

2003

Oliveria-Brett O., Silva LA., Farace G., Vadgama P., Brett CMA. Voltammetric and impedance studies of iosone-5'-monophosphate and hypoxanthine. Bioelectrochmistry 59, 49-56

2002

Farace G., Lillie, G., Hianik T., Payne P., Vadgama P. Reagentless biosensing using electrochemical impedance spectroscopy. Bioelectrochmistry 55, 1-3

Gill A., Farace, F., Lillie G., Vadgama P. Strategic issues in reliable sensing. Bioelectrochmistry 55, 123-125

2001

Farace G., Vadgama P. Basica structure and functional properties of medical biosensors with special regard to biocompatibility. Biocybernetics and Biomedical Engineering 21, 11-20

Wilde LM., Farace G., Roberts CJ., Davies, MC., Sanders, GHW., Tendler SJB., Williams PM. Molecular patterning on carbon based surfaces through photobiotin activation. Analyst 126, 195-198

Conference Abstracts

2009

Correlation of N-terminal prohormone brain natriuretic peptide with left ventricular outflow tract in dogs with sub aortic stenosis. ACVIM 27th Annual Forum

Pulmonary hypertension and N-terminal prohormone brain natriuretic peptide in dogs. ACVIM 27th Annual Forum

2008

Effect of arrythmias on natriuretic peptide levels in dogs with wither valve disease or with structurally normal hearts. ECVIM 18th Congress

Troponin Concentrations in Patients with Masses or Tumors. ACVIM 26th Annual Forum

2002

The use of microfluidics to reduce biofouling at flow-through biosensors. 2nd Workshop of the Concerted Action: Evaluation/Validation of Novel Biosensors in Real Environmental and Food Samples

2001

Electrochemical impedance spectroscopy (EIS) for reagentless bioaffinity sensing. 16th Conference of the European Society of Biomaterials

Preliminary electrochemical and optical detection methodologies for detection of hormones. 1st Workshop of the Concerted Action: Evaluation/Validation of Novel Biosensors in Real Environmental and Food Samples

EXHIBIT B

Aim: To determine if human NTproBNP can be measured using a canine specific NTproBNP assay.

Methods: Human glycosylated NTproBNP (Cat#: 8NT1, Lot 05/11-8TN1) and Human NTproBNP (Cat#:8GOB2, Lot 07/06-8GOB2) were obtained from Hytest Ltd (Finland) and diluted in BNP-free human plasma (Cat# 8FBP. Lot 06/01-8BFP, Hytest Ltd). Dilutions were made serially from 100ng/ml down to 0.78ng/ml.

These were then run on a canine NTproBNP plate from Guildhay Ltd, UK (VetSign Canine Cardioscreen NTproBNP, VC4010) using the method described on the kit insert. The only modifications being that the human samples were run in triplicate rather than duplicate and the standard curve was fitted using a linear fit.

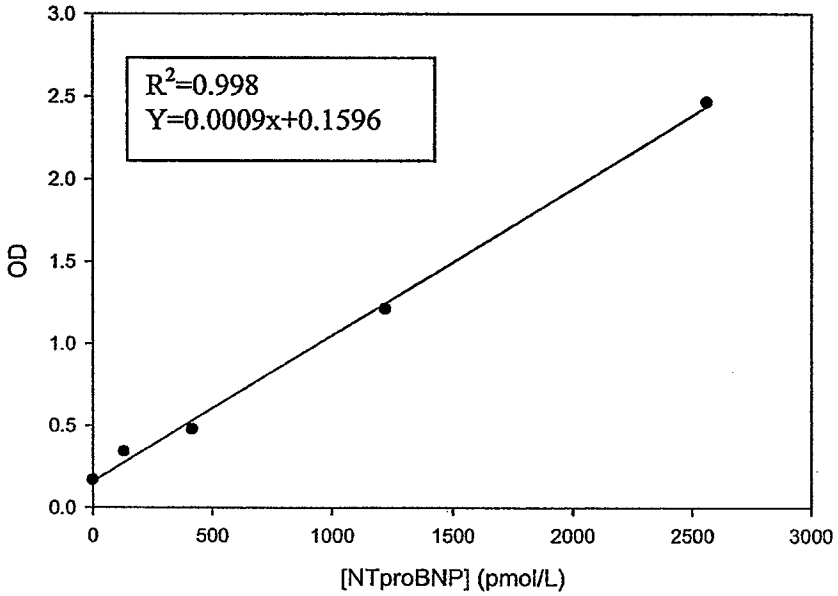
	1	2	3	4	5	6	7	8	9	10	11	12
A	100ng/ml NTproBNP	25ng/ml NTproBNP	6.25ng/ml NTproBNP	1.56ng/ml NTproBNP	0ng/ml NTproBNP	50ng/ml NTproBNP Glycosylated	12.5ng/ml NTproBNP Glycosylated	3.12ng/ml NTproBNP Glycosylated	0.78ng/ml NTproBNP Glycosylated			
B	100ng/ml NTproBNP	25ng/ml NTproBNP	6.25ng/ml NTproBNP	1.56ng/ml NTproBNP	0ng/ml NTproBNP	50ng/ml NTproBNP Glycosylated	12.5ng/ml NTproBNP Glycosylated	3.12ng/ml NTproBNP Glycosylated	0.78ng/ml NTproBNP Glycosylated			
C	100ng/ml NTproBNP	25ng/ml NTproBNP	6.25ng/ml NTproBNP	1.56ng/ml NTproBNP	0ng/ml NTproBNP	50ng/ml NTproBNP Glycosylated	12.5ng/ml NTproBNP Glycosylated	3.12ng/ml NTproBNP Glycosylated	0.78ng/ml NTproBNP Glycosylated			
D	50ng/ml NTproBNP	3.12ng/ml NTproBNP	12.5ng/ml NTproBNP	0.78ng/ml NTproBNP	100ng/ml NTproBNP Glycosylated	25ng/ml NTproBNP Glycosylated	6.25ng/ml NTproBNP Glycosylated	1.56ng/ml NTproBNP Glycosylated	0ng/ml NTproBNP Glycosylated			
E	50ng/ml NTproBNP	3.12ng/ml NTproBNP	12.5ng/ml NTproBNP	0.78ng/ml NTproBNP	100ng/ml NTproBNP Glycosylated	25ng/ml NTproBNP Glycosylated	6.25ng/ml NTproBNP Glycosylated	1.56ng/ml NTproBNP Glycosylated	0ng/ml NTproBNP Glycosylated			
F	50ng/ml NTproBNP	3.12ng/ml NTproBNP	12.5ng/ml NTproBNP	0.78ng/ml NTproBNP	100ng/ml NTproBNP Glycosylated	25ng/ml NTproBNP Glycosylated	6.25ng/ml NTproBNP Glycosylated	1.56ng/ml NTproBNP Glycosylated	0ng/ml NTproBNP Glycosylated			
G	Standard 5	Standard 4	Standard 3	Standard 2	Standard 1							
H	Standard 5	Standard 4	Standard 3	Standard 2	Standard 1							

Results:

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.174	0.158	0.138	0.181	0.138	0.176	0.205	0.150	0.145			
B	0.173	0.157	0.135	0.197	0.134	0.174	0.200	0.139	0.155			
C	0.191	0.169	0.138	0.224	0.135	0.172	0.201	0.145	0.161			
D	0.182	0.144	0.139	0.178	0.135	0.161	0.188	0.137	0.183			
E	0.178	0.138	0.140	0.218	0.139	0.183	0.193	0.144	0.165			
F	0.170	0.133	0.145	0.181	0.136	0.164	0.206	0.133	0.159			
G	2.437	1.060	0.401	0.336	0.125							
H	2.491	1.359	0.549	0.342	0.206							

Standards

Standard	Average OD
1 – 0pmol/L	0.166
2 – 131pmol/L	0.339
3 - 417pmol/L	0.475
4 – 1220pmol/L	1.210
5 – 2561pmol/L	2.464



Human Spiked Samples

Expected Concentrations		Human NTproBNP		Glycosylated Human NTproBNP	
ng/ml	pmol/L	Average OD	Calculated Concentration (pmol/L)	OD	Calculated Concentration (pmol/L)
100ng/ml	11765	0.179	21.9	0.137	<0
50ng/ml	5882	0.177	19.0	0.174	16.0
25ng/ml	2941	0.161	1.9	0169	10.8
12.5ng/ml	1471	0.141	<0	0.202	47.1
6.25ng/ml	735	0.137	<0	0.196	40.1
3.12ng/ml	367	0.138	<0	0.145	<0
1.56ng/ml	184	0.201	45.6	0.138	<0
0.78ng/ml	92	0.192	36.4	0.154	<0
0ng/ml	-	0.136	<0	0.169	10.4

Conclusion:

The canine kit does not appear to recognize human NTproBNP in either its glycoylated or its non-glycoylated form.

EXHIBIT C

Aim: To determine if canine calibrators and feline calibrators cross react such that they can be read on the opposite species kit (i.e. canine on feline and vice versa).

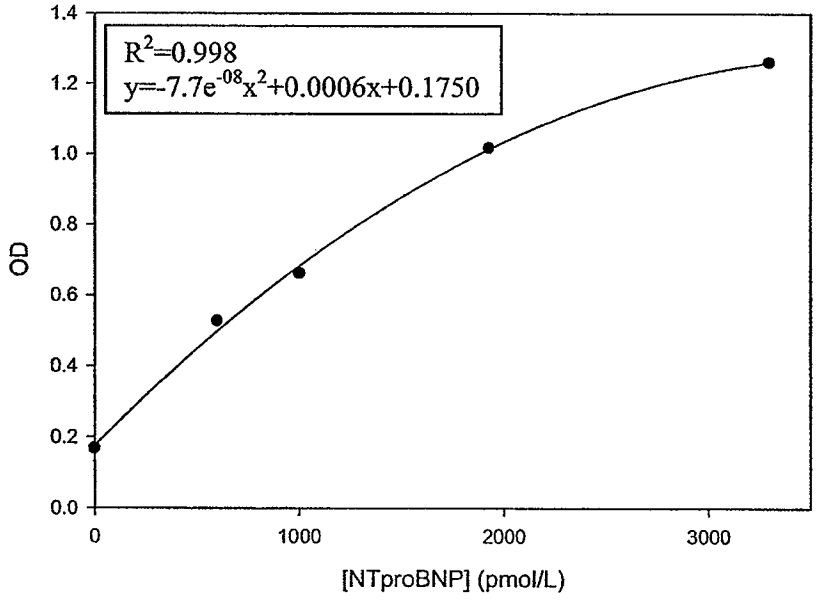
Methods: Calibrators from the two species specific kits (canine – KE970, IDEXX Laboratories, Inc, USA and feline –VC5010-9G Biomedica Gruppe, Austria) were run on the opposite species kit. The kits were run according to IDEXX SOP for the 5 hour assay and each case the standard curve was fitted using a quadratic fit.

Results:

Standards

Canine

Standard	Average OD
1 – 0pmol/L	0.167
2 – 602pmol/L	0.526
3 - 1003pmol/L	0.661
4 – 1928pmol/L	1.016
5 – 3296pmol/L	1.260

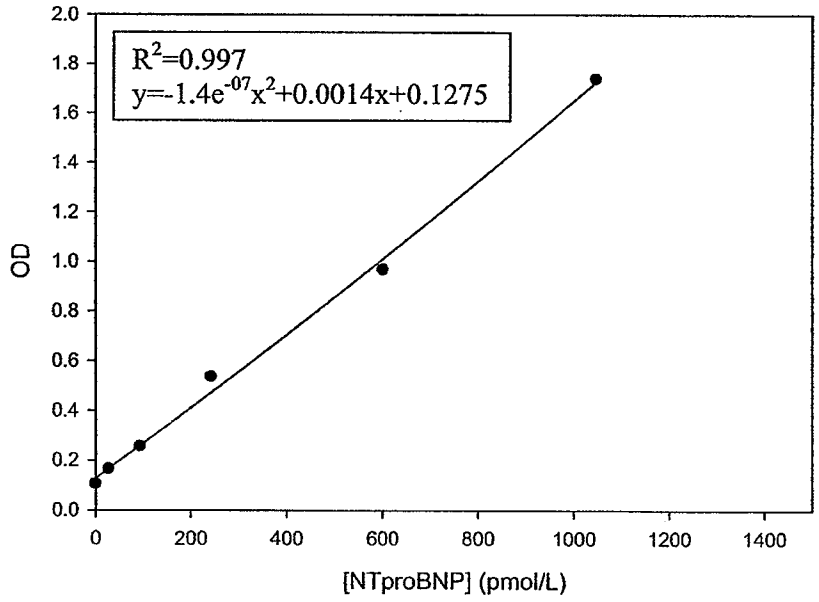


Feline Calibrators

Calibrator	Average OD	Calculated Concentration (pmol/L)	Expected Concentration (pmol/L)
1	0.113	0	0
2	0.109	0	27
3	0.103	0	94
4	0.111	0	243
5	0.110	0	602
6	0.106	0	1047

Feline

Standard	Average OD
1 – 0pmol/L	0.104
2 – 27pmol/L	0.163
3 – 94pmol/L	0.254
4 – 243pmol/L	0.535
5 – 602pmol/L	0.966
6 – 1047pmol/L	1.738



Canine Calibrators

Calibrator	Average OD	Calculated Concentration (pmol/L)	Expected Concentration (pmol/L)
1	0.095	0	0
2	0.094	0	602
3	0.088	0	1003
4	0.097	0	1928
5	0.089	0	3296

Conclusion:

Canine NTproBNP calibrators are not recognized on a feline specific kit and feline NTproBNP calibrators are not recognized on a canine specific kit.

EXHIBIT D

VETSIGN™
Canine CardioSCREEN
Nt-proBNP

For the Quantitative Measurement of Nt-proBNP in Canine Serum & Plasma

Product Code: VC4010

Principle of the assay

The importance of natriuretic peptides in the diagnosis and prognosis of heart disease and failure is well demonstrated in Humans. Studies undertaken by Guildhay and its collaborators have shown that natriuretic peptides have similar value in the diagnosis of dogs with heart disease and heart failure.

As well as being an efficient pump the heart is also an important endocrine organ. As part of its endocrine function, cardiomyocytes in the heart muscle produce and secrete a family of related peptide hormones, called Natriuretic Peptides (NPs). The release of NPs is greatly increased in diseases characterized by an expanded fluid volume, including heart failure (HF), renal failure, and liver cirrhosis. The NPs are natural antagonists to the renin-angiotensin-aldosterone system (RAAS), and their role in regulating fluid balance appears to be particularly important in patients with haemodynamic stress such as heart failure.

It has now been demonstrated through research that BNP is principally secreted from the heart and is secreted mainly in the left ventricles. As the ventricles of the compensated heart cells stretch (wall stress), BNP is synthesized as a prohormone proBNP and is secreted into the blood stream and is cleaved into NT-proBNP and BNP. However because BNP has a short plasma half-life, it also has lower concentrations than Nt-proBNP. Guildhay has developed the assay to detect Nt-proBNP because in canine circulation, plasma BNP levels decline in a biphasic manner with a half life of 1.57 ±0.14 minutes and a terminal half life of 301 ± 85 minutes. (shorter half life than in rats human and sheep).

This test kit is a sandwich EIA (Enzyme Immuno Assay) designed to measure the immunoreactive Nt-proBNP in canine serum and plasma samples. To achieve high specificity the kit incorporates two immunoaffinity purified sheep antibodies specific for Canine Nt-proBNP. The plate consists of the capture antibody anti-Nt-proBNP (25-41) bound to the wells of the plate. The Tracer comprises the detection antibody, anti-Nt-proBNP (1-22) conjugated to horseradish peroxidase.

In the incubation step, standard or sample and conjugated detection antibody (Tracer) are added simultaneously to the wells. Nt-proBNP if present in the sample binds to the capture antibody precoated in the wells and forms a sandwich with the detection antibody.

After a washing step which removes any non specific bound material, substrate (TMB) is added to the wells. Bound Nt-proBNP is quantified by an enzyme catalysed colour change detectable on a standard microtitre plate reader. The amount of colour developed is directly proportional to the amount of Nt-proBNP immunoreactivity present in the standard or sample. A standard curve is plotted from the values measured and the concentration of Nt-proBNP in the samples is calculated from this curve.

Contents of the kit

1. 12 x 8 well microtitre strips in strip holder, packed in a sealed foil bag with desiccant. Wells are coated with an affinity purified polyclonal sheep anti Nt-proBNP (25-41) antibody.

2. 10x Concentrated wash buffer concentrate. The bottle contains 100 ml of wash buffer concentrate.

3. Tracer. The vial contains 25 ml affinity purified polyclonal sheep anti Nt-proBNP (1-22) antibody conjugated to horseradish peroxidase (HRP), in red stabiliser solution.

4. 5 standards ranging from 0 to ~3000 pmol/L (white caps). The vials contain synthetic canine Nt-proBNP lyophilised in serum, which when reconstituted make up to 0.3mL. The concentration of each standard after reconstitution is stated on the label.

5 Substrate. The vial contains 25 ml TMB solution, ready for use.

6. Stop solution. The vial contains 10 ml of stop solution, ready for use.

7. 2 self-adhesive plastic films.

8. Protocol sheet.

9. Instructions for use (kit insert).

10. Quality Control Certificate.

Additional materials and equipment required

- Distilled water
- Variable volume pipettes in the range of 20 µl to 1000 µl
- Multichannel pipette or multipette
- Manual or automatic microplate washer
- Microtitre plate reader equipped with 450 nm filter
- Graph paper or software for calculation of results
- *Optional: incubator at 23°C*

Guide to Interpretation of Results and Findings from Validation Study:

The following recommendations are made for the interpretation of results:

1. It is recommended that results are report only as whole numbers.

2. All samples reading less than 42 pmol/L should be reported as less than 42pmol/L as this is the limit of detection for the kit.

3. The following are some examples of potential unexpected results and their interpretation:

Result		Interpretation
< 210 pmol/L	⇒	No indication of Cardiac Disease
≥210 & < 300 pmol/L	⇒	High normal, probable Cardiac Disease (>86%), consider further work up and re-test Nt-proBNP < 3 months.
≥ 300 pmol/L	⇒	>99% Certain Cardiac Disease.

Unexpected Results		
0 pmol/L	⇒	Indicates poor sample handling. For a sample with a result of 0 a repeat sample is required.
<42 pmol/L	⇒	If the dog is symptomatic this indicates probable poor sample handling. Repeat the test and/or re-sample.
≥ 42 & ≤ 210 pmol/L	⇒	If symptomatic possible incorrect sample handling repeat test and/or re-sample.

4. Trial Findings

Comparing cases where a diagnosis of mitral regurgitation was made by the attending clinician to those in which a diagnosis of dilated cardiomyopathy was made Nt-proBNP level were significantly higher in dogs with dilated cardiomyopathy (p=0.001).

Elevation of proBNP peptide is greater in dogs with primary myocardial disease than those with mitral valve disease, although dogs with mitral disease still show elevations when compared to normal animals or those with respiratory disease.

Plasma and serum Nt-proBNP can be used to accurately discriminate animals with heart disease and failure from animals with other respiratory signs due to non-cardiac disease.

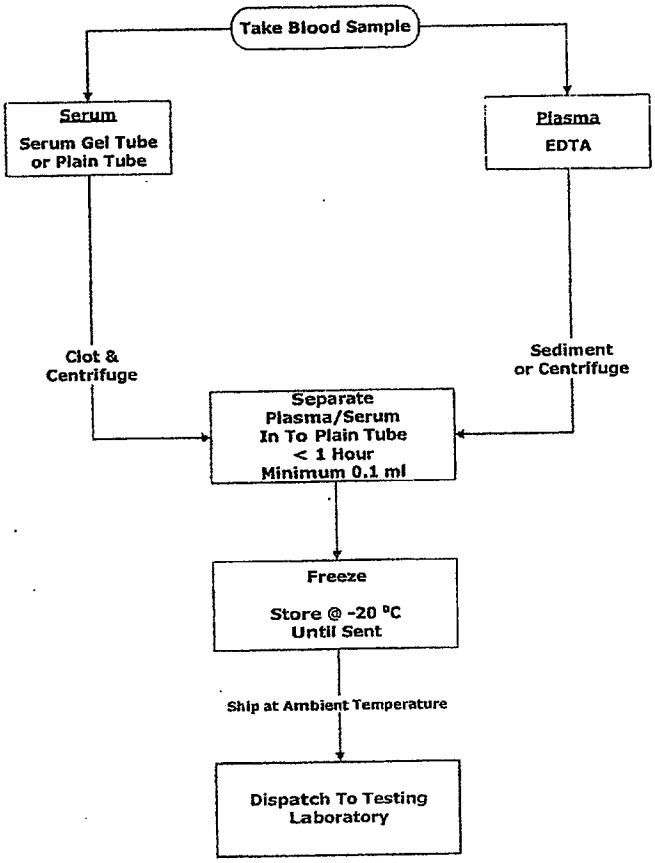
Sample Handling

The test requires either a serum or plasma sample. The correct handling of the sample, at the veterinary practice and the laboratory can have a significant impact on the result obtained (see troubleshooting guide)

For the benefit of clarity we have included detailed guides for both the Veterinarian and the Laboratory to adopt:

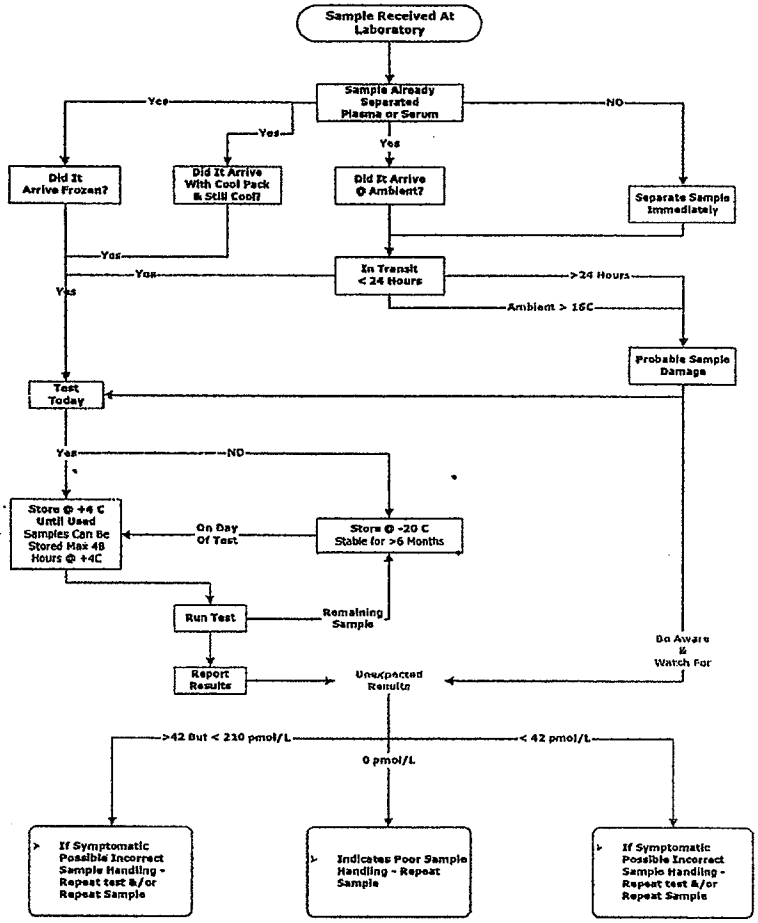
I. Sample Collection Guide for the Veterinarian:

- **Take blood (≈ 2ml) into a plain (no-anticoagulant) tube *or* EDTA tube.**
- **Label with the date and ID of the dog.**
- **Leave the blood to either:**
  - **⇒ Clot for approximately 30 minutes ideally at +4°C, or**
  - **⇒ Red Blood Cells to sediment 30 minutes ideally at +4°C**
- **Separate the serum or plasma, by centrifugation if not already clearly separated by standing/sedimentation.**
  - **⇒ Centrifuge at (≈1500g) for 5-10 minutes ensuring that after centrifugation there is a clear distinction between the cells and the serum/plasma (for serum, if this has not happened re-centrifuge, as the most likely cause is incomplete clotting).**
- **Transfer the serum or plasma into a second (plain) sample tube. Laboratory needs approximately 100 µL / 0.1 ml of Serum or Plasma for each test. Label clearly.**
- **Store Sample at -20°C or +2 to 8°C until sent to laboratory for testing. If to be stored for >24 hours freeze the sample.**


Take Blood Sample
Serum
Serum Gel Tube
or Plain Tube
Plasma
EDTA
Clot &
Centrifuge
Sediment
or Centrifuge
Separate
Plasma/Serum
In To Plain Tube
< 1 Hour
Minimum 0.1 ml
Freeze
Store @ -20 °C
Until Sent
Ship at Ambient Temperature
Dispatch To Testing
Laboratory

1. Sample Collection Guide for the Laboratory:

- **Check that sample is already separated i.e. off the clot/RBC's and as serum or plasma. If not, separate sample immediately.**
  - ⇒ **If not separated and sample has been in transit for >24 Hours probable sample damage.**
  - ⇒ **If sample in transit <24 hours results may be valid.**
- **If sample transit time <24 hours and sample was separated and arrived cooled, frozen or if ambient temperature averaged < 16°C sample should be valid.**
  - ⇒ **If in transit for >24 hours during high ambient temperatures sample results may not be valid.**
- **Analyse Today?**
  - **YES** **If sample to be analysed on day or receipt, store at +4°C until tested, then store remaining sample at -20°C. Frozen sample are stable for > 6 months.**
  - **NO** **Store at -20°C until day of test, thaw on day of test, then store remaining sample at -20°C. Frozen sample are stable for > 6 months.**


Sample Received At Laboratory
Sample Already Separated Plasma or Serum
Yes
Yes
Yes
NO
Did It Arrive Frozen?
Did It Arrive With Cool Pack & Still Cool?
Did It Arrive @ Ambient?
Separate Sample Immediately
Yes
Yes
In Transit < 24 Hours
>24 Hours
Yes
Ambient > 16C
Probable Sample Damage
Test Today
Yes
NO
Store @ +4 C Until Used Samples Can Be Stored Max 48 Hours @ +4C
On Day Of Test
Store @ -20 C Stable for >6 Months
Run Test
Remaining Sample
Be Aware & Watch For
Report Results
Unexpected Results
>42 But < 210 pmol/L
< 42 pmol/L
0 pmol/L
If Symptomatic Possible Incorrect Sample Handling - Repeat test &/or Repeat Sample
Indicates Poor Sample Handling - Repeat Sample
If Symptomatic Possible Incorrect Sample Handling - Repeat test &/or Repeat Sample

K9 proBNP

Reagent and sample preparation

- Sample handling is important, for optimal results:
  1. Plasma or serum should be separated from blood samples as soon as possible after collection.
  2. Plasma or serum should be frozen (-20°C) until analysed. See sample handling guide.
- Lipaemic or haemolytic samples may give erroneous results.
- Samples should be mixed well before assaying.
- When first using the test and until familiar with the process it is recommended that samples are run as duplicates, thereafter samples may be run as singles but where unexpected results occur a re-run in duplicate is advised.
- Mark positions for standards and samples on the protocol sheet supplied.
- Take microtitre strips out of the bag and mark as appropriate. Store unused strips with the desiccant at 4°C in the foil bag.
- Allow all reagents and samples to reach room temperature (18-26°C) before use in the assay.

Precautions

- All liquid reagents contain 0.01% Proclin300 as preservative.
- ProClin™300 is not toxic in concentrations used in this kit but may cause allergic skin reactions. Avoid contact with skin and eyes.
- Do not pipette by mouth.
- Avoid all contact with the reagents by using gloves.
- Stop solution contains diluted sulphuric acid. Irritation to eyes and skin is possible. Flush with water after contact!

Assay Instructions

1. Dilute wash buffer concentrate to 1000 ml (add 100 ml of concentrate to 900 ml of distilled water) mix well. Crystals in the buffer concentrate will dissolve at room temperature. Buffer is stable at 4°C until expiry date stated on the label.

2. Dissolve freeze dried standards in 0.3 ml of distilled water and leave at room temperature (18-26°C) for 30 min., mix well. For storage, freeze the standards at –20°C, these standards are stable until quoted expiry date.

3. Add 20 µl of each standard concentration and samples into the respective wells.

4. Add 200 µl of tracer (red) to all wells, mix well.

5. Cover strips with plastic film and incubate in the dark overnight (16-24 hours) at room temperature (18-26°C) or if available at 23°C in an incubator.
   Ensure all wells are sealed well with the film to avoid evaporation.

6. Discard contents of the wells and wash 5x with 350 µl diluted wash buffer. Remove any remaining wash buffer by hitting plate against paper towel after the last wash.

7. Add 200µl substrate to all wells, shake well.

8. Cover strips with plastic film and incubate in the dark for 30 min at room temperature (18-26°C) or if available at 23°C in an incubator. Ensure all wells are sealed well with the film to avoid evaporation.

9. Add 50 µl of stop solution to all wells and mix well. Determine absorbance immediately with a microtitre plate reader at 450 nm. If the absorbance of the highest standard exceeds the measuring range of the photometer, the assay should be re-measured immediately at 405 nm

Assay Incubation Scheme Summary

	Standard Wells	Sample Wells
Standard	20 µl	-
Sample	-	20 µl
Tracer (A/B-HRP)	200 µl	200 µl

- Incubate the microplate strips covered with plastic film overnight (16 - 24 hours) at 23°C or at room temperature (18 - 26°C) in the dark.
- Discard contents of the wells and wash x5 with 350 µl diluted wash buffer. Remove any remaining wash buffer by hitting plate against paper towel after the last wash.

Substrate	200 µl	200 µl

- Incubate the microplate strips covered with plastic film for 30 min. at 23°C or at room temperature (18 to 26°C) in the dark.

Stop Solution	50 µl	50 µl

- Mix, read absorption with an ELISA reader at 450 nm
- If the absorbance of the highest standard exceeds the measuring range of the photometer, the assay should be re-measured immediately at 405 nm.

Calculation and Interpretation of results

A calibration curve of Absorbance against Standard concentration can be plotted manually on graph paper. Alternatively, the curve may be plotted using commercially available curve fitting software such as Spline or 4PL algorithm. Results of the samples are read from this calibration curve. If the concentration of the samples is above the standard range, a dilution of samples is recommended.

	Serum or Plasma Sample	Sensitivity	Specificity
Negative: No indication of Heart Disease or Heart Failure	< 210 pmol/L	85%	90%
High Normal: In the absence of clear clinical symptoms re-test in 3-6 months	≥ 210 pmol/L and < 300 pmol/L		
Heart Disease/Failure: Where symptoms evident at clinical examination	≥ 300 pmol/L	45%	100%

NOTE:
The Canine CardioSCREEN Nt-proBNP test is not in itself a definitive diagnostic test and the results, as with other individual assay results, should be viewed in conjunction with other diagnostic information.

Technical hints

- To avoid cross-contamination, change pipette tips between addition of standards, samples, tracer, substrate and stop solution. Also use separate reservoirs for each reagent!
- Do not mix stoppers and caps of different reagents, this causes contamination!
- Do not use reagents beyond expiry date and Protect reagents from direct sunlight.
- Do not mix or substitute reagents with those from other lots or sources except for wash buffer concentrate.
- Gently tap the standard on the bench prior to reconstitution to remove any lyophilised material from the bung.
- Post addition of water to the standards roll the sample to improve reconstitution.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- To ensure efficient washing leave wash buffer in the wells for a minimum of 10 seconds before aspiration.
- Substrate solution should remain colourless until added to the plate.
- Stop solution should be added to the plate in the same order as the substrate solution.
- For accurate results a spline or 4 parameter logistic curve fitting algorithm must be used for the interpretation of results.

Troubleshooting Guide

Symptom	Possible Cause	Recommended Action
1. After plotting my Standard Curve when I read off the results for samples I get a value of 0 (zero)	A zero result is not probable even with the healthiest of dogs. If this result is obtained it is indicative of incorrect sample handling. Probable causes being: a. Sample (serum or plasma) not separated from red blood cells within 24 hours of taking sample. b. Sample held at ambient (18-26°C) for more than 24 hours.	A new sample is required for testing. Ensure that the sample is collected and handled in accordance with the sample collection instructions of these Instructions For Use. These can also be obtained from Guildhay or via the Guildhay web site.
2. After plotting my Standard Curve when I read off the results for samples I get a negative value.	Use of the wrong type of curve fit e.g. linear regression. This can make samples that have a greater OD than the standard 1 (0 pmol/L) read as negative.	Recalculate the sample values against the standard curve fitted by either Spline or 4 parameter logistic curve fits.
3. My low standards give higher than expected ODs	Non-specific binding in the assay where conjugated secondary antibody has not been washed from the wells	Ensure that a there is a 10 second soak step in the wash step of the assay.

Assay Validation

Intra Assay Precision:

Low	20	Mean % CV	362.8 6	370.4 8.5	347.5 4.9	360.2 6.4
Med	20	Mean % CV	665.6 9.8	652.8 6.6	682.0 8.8	666.8 8.4
High	20	Mean % CV	1670.0 7.5	1722.0 9.2	1838.7 4.7	1743.6 7.1

Inter Assay Precision:

Low	60	360.2	25.6	7.1
Med	60	666.8	57.1	8.6
High	60	1743.6	143.5	8.2

Minimum detectable dose (limit of detection)

	10	0.166	0.017	0.217	42

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EXHIBIT E

ASSAY INSTRUCTIONS FOR CANINE CARDIOSCREEN NT-PROBNP ASSAY

1) Dilute wash buffer concentrate to 1000ml (add 100ml of concentrate to 900ml of distilled water) mix well. Crystals in the buffer concentrate will dissolve at room temperature. Buffer is stable at 4°C until expiry date stated on the label.

2) Reconstitute lyophilized standards in 0.3 ml of distilled water and leave at room temperature for 30 minutes, mix well. These standards when stored at -20°C are stable until quoted expiry date.

3) Add 30µl of each standard concentration and samples into the respective wells. [NOTE: Standards and samples are added to side of well, not center.]

4) Add 200µl of tracer (red) to all wells. Mix well. [NOTE: Mix by tapping side of plate several times, not aspirating into pipette.]

5) Cover strips with plastic film and incubate in the dark for 5 hrs at 23°C in an incubator or at room temperature (18-26°C). Ensure all wells are sealed well with the film to avoid evaporation.

6) Discard contents of wells and wash 5X with 350µl diluted wash buffer. Remove any remaining wash buffer by hitting plate against paper towel after the last wash. [NOTE: All washes to be performed using Skatron plate washer.]

7) Add 200µl substrate to all wells; shake well.

8) Cover strips with plastic film and incubate in the dark for 40 min at 23°C in an incubator or at room temperature (18-26°C). Ensure all wells are sealed well with the film to avoid evaporation.

9) Add 50µl stop solution to all wells and mix well. Determine absorbance immediately with an ELISA reader at 450nm. If the absorbance of the highest standard exceeds the measuring range of the photometer, the assay should be re-measured immediately at 405nm.

10) Calculate slope and y-intercept of standards using 450nm data. Determine [pmol/L] values using slope and y-intercept from standards.

EXHIBIT F

ASSAY INSTRUCTIONS FOR FELINE CARDIOSCREEN NT-PROBNP ASSAY

1) Dilute wash buffer concentrate to 1000ml (add 100ml of concentrate to 900ml of distilled water) mix well. Crystals in the buffer concentrate will dissolve at room temperature. Buffer is stable at 4°C until expiry date stated on the label.

2) The standards are in liquid form and ready to use. These standards when stored at 2-8°C are stable until quoted expiry date.

3) Add 30µl of each standard concentration and samples into the respective wells. [NOTE: Standards and samples are added to side of well, not center.]

4) Add 200µl of tracer (red) to all wells. Mix well. [NOTE: Mix by tapping side of plate several times, not aspirating into pipette.]

5) Cover strips with plastic film and incubate in the dark for 5 hrs at 23°C in an incubator or at room temperature (18-26°C). Ensure all wells are sealed well with the film to avoid evaporation.

6) Discard contents of wells and wash 5X with 350µl diluted wash buffer. Remove any remaining wash buffer by hitting plate against paper towel after the last wash. [NOTE: All washes to be performed using Skatron plate washer.]

7) Add 200µl substrate to all wells; shake well.

8) Cover strips with plastic film and incubate in the dark for 40 min at 23°C in an incubator or at room temperature (18-26°C). Ensure all wells are sealed well with the film to avoid evaporation.

9) Add 50µl stop solution to all wells and mix well. Determine absorbance immediately with an ELISA reader at 450nm. If the absorbance of the highest standard exceeds the measuring range of the photometer, the assay should be re-measured immediately at 405nm.

10) Calculate slope and y-intercept of standards using 450nm data. Determine [pmol/L] values using slope and y-intercept from standards.